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The purpose of this study is to identify integrin-specific signalling events that may be responsible for development of the malignant phenotype in mammary epithelium. Our laboratory has focused on a novel ligand for epithelial integrins, laminin-5r, which is located in mammary epithelial basement membrane. We hypothesize that binding of integrins to laminin-5r elicits downstream signalling events that may help define the normal phenotype of mammary epithelial cells; disruption of these signals may contribute to the development of malignancy. To date we have succeeded in raising six monoclonal antibodies against laminin-5r, and have used them to define distinct structural conformations of laminin-5r using a combination of immunoprecipitation, western blot, and elisa assays. Also, we have used these antibodies to affinity purify laminin-5r from conditioned medium of a rat epithelial cell line, 804G. Finally, we have shown that one of these antibodies blocks adhesion of a variety of cells, including mammary epithelial cells, to purified laminin-5r.

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Introduction

The long-range goal of this study is to determine the contribution of specific integrin receptors to the development of the malignant phenotype in mammary epithelial cells. Our working hypothesis is that normal mammary epithelial cells utilize specific integrin receptors to establish stable contacts with specific extracellular matrix (ECM)[1] molecules in the underlying basement membrane, and that biochemical signals accompanying the formation of these contacts play a critical role in maintaining the normal phenotype. We predict that malignant epithelial cells exhibit changes in these biochemical signaling pathways. Our more immediate goal is to identify and characterize specific components of these signaling pathways in both normal and malignant mammary epithelial cells.

Cell adhesion to ECM is required for proper control of cell growth and function. Cells bind ECM molecules through specific receptors. A majority of these receptors belong to the integrin family of cell surface adhesion molecules. Integrins are heterodimers, consisting of one α and one β subunit; at present, at least 14α and 8β integrin subunits have been identified, which organize into at least 20 different receptors that bind a wide variety of ECM and cell surface molecules (Hynes, 1992).

One prominent class of ECM molecules are the laminins. Laminins are a family of ECM molecules found in the basement membrane of epithelial sheets. Laminins promote adhesion and migration of epithelial cells and may play a role in tumor progression and metastasis (Tryggvason, 1993; Kibbey et al., 1994; Sweeney et al., 1991). Laminin molecules form characteristic cross-shaped heterotrimers consisting of one α, one β, and one γ chain arranged as an α helical, coiled coil "core" with three short arms (Tryggvason, 1993; Yurchenco and Cheng, 1994); at present, at least 3α, 3β and 2γ subunits have been identified, which organize into at least 7 different laminin isoforms. A number of integrin heterodimers have been identified as potential receptors for laminins, including α1β1, α2β1, α3β1, α6β1, α7β1, and α6β4 (Sonnenberg, 1993).

A current focus of this lab has been on an isoform of laminin that we have named laminin-5r. Laminin-5r is derived from the rat bladder carcinoma cell line 804G. Immunostaining with polyclonal and monoclonal antibodies directed against laminin-5r indicates that it is present in the basement membranes of a wide variety of epithelial tissues including skin, gut, and mammary gland (e.g., Fig. 1). We have previously shown

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[1]Abbreviations used: BSA, bovine serum albumin; ECM, extracellular matrix; PBS, phosphate buffered saline; PBST,PBS/0.2%Tween 20/0.01%thimerosal; PVDF, polyvinylidene difluoride; RIPA, radioimmunoprecipitation assay; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS polyacrylamide gel electrophoresis.

that ECM derived from 804G cells supports rapid adhesion and spreading of human keratinocytes and promotes hemidesmosomes in these cells (Langhofer et al., 1993). Immunostaining of human keratinocytes plated on 804G matrix has shown that laminin-5r and the human α6 integrin codistribute in a pattern reminiscent of that observed for markers of hemidesmosomes (Langhofer et al., 1993), raising the possibility that laminin-5r may play an important role in the assembly of stable adhesion complexes between epithelial cells and their underlying basement membrane. Importantly, plating of human keratinocytes on human laminin-5 or laminin-5r promotes more efficient cell adhesion and spreading when compared to laminin-1 (Rousselle and Aumailley, 1994)[2]. Laminin-5 therefore likely represents a more physiologically relevant ligand than laminin-1 for integrins in epithelial cells.

Consequently, the focus of project outlined in the original proposal for this grant has shifted away from laminin-1 and towards laminin-5r. Accordingly, the objectives of the first year were modified so as to allow for isolation, purification, and partial characterization of laminin-5r prior to embarking upon studies of integrin-mediated signalling accompanying adhesion to laminin-5r. Also, the specific aims for the second year have been modified to reflect the inclusion of laminin-5r in this study.

Body

Materials and Methods

Cells. Rat 804G and human FGmet2 carcinoma cells were routinely passaged in DMEM or RPMI medium (Gibco, Grand Island NY), respectively, supplemented with 10% fetal calf serum (Gemini, Irvine CA) and 2 mM glutamine (BioWhittaker, Walkersville MD) at 37°C in a humidified incubator containing 5% CO_2. Human SCC12 carcinoma cells were routinely passaged in Keratinocyte Growth Medium (Clonetics, San Diego CA) lacking gentamycin. Human SCC25 carcinoma cells were grown in media containing 3:1 DMEM:Ham's F12 medium (ICN, Costa Mesa CA) supplemented with 0.4 mg/ml hydrocortisone (Sigma, St. Louis MO), 2 mM glutamine, and 10% fetal calf serum. 804G and FGmet2 cells were harvested for experiments by washing confluent plates with phosphate buffered saline (PBS) and incubating them in trypsin/versene (BioWhittaker) for 10 min at 37°C. Primary normal mammary epithelial cells were obtained from Clonetics and routinely passaged in Epithelial Cell Growth Medium (Clonetics). MCF7, MDA-MB-231, and MDA-MB-435 cells were passaged in RPMI medium supplemented with 10% fetal calf serum. Following typsinization, 804G, FGmet2, MCF7, MDA-MB-231, and MDA-MB-435 cells were suspended in culture medium containing

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[2]Hormia, M., J. Falk-Marzillier, G. Plopper, R.N. Tamura, J.C.R. Jones, and V. Quaranta. (1995) Rapid spreading and mature hemidesmosome formation in HaCaT keratinocytes induced by incubation with soluble laminin-5r. J. Invest. Dermatol. (in press).

10% fetal calf serum to neutralize trypsin, centrifuged, and suspended in fresh culture medium. SCC12 and primary mammary epithelial cells were harvested and passaged using the Trypsin System (Clonetics) as indicated by the manufacturer.

Collection of cell-associated laminin -5r. 804G cells were grown to confluency either on 96 well plates or on 150 mm plastic petri dishes. The culture medium was removed and the cells were washed in sterile PBS. The cells were removed according to the method of Gospodarowicz (Gospodarowicz, 1984) by incubating them 2 x 5 min in 20 mM sterile NH_4OH. The plates were extensively washed with PBS and distilled water and allowed to air dry. For recovering 804G matrix, each 150 mm Petri dish was incubated with 1 ml of collection buffer (10 mM Tris-HCl, pH 7.0, 0.1 % sodium dodecyl sulfate [SDS], 100 mM β-mercaptoethanol). After 15 min at 37°C the fluid was collected with a cell scraper and lyophilized. The matrix pellets were solubilized either directly into Laemmli sample buffer or collection buffer. Matrix was dialyzed against PBS prior to immunoprecipitation.

Western blotting. Protein samples diluted in 2x sample buffer containing 50 mM dithiothreitol and 1.5M β mercaptoethanol were heated 5 min at 95°C then separated by SDS-PAGE using 6% polyacrylamide gels (Novex, San Diego CA). Separated proteins were transferred to PVDF membranes (Biorad, Hercules CA) using a Biorad Transwell system. Protein bands were visualized using Ponceau S stain (Sigma), then membranes were cut in strips and blocked 1 hr with blotto (5% nonfat dried milk in PBS/0.2% Tween 20/0.01% antifoam A [Sigma]/0.01% thimerosal). Primary antibodies diluted 1:1000 in blotto were added for 1 h at room temperature, then the strips were washed 2x with PBS/0.2%Tween 20/0.01%thimerosal (PBST). Secondary antibodies (goat anti-mouse IgG or anti-rabbit IgG, conjugated to alkaline phosphatase; Promega, Madison WI) were diluted 1:1000 in blotto and added for 1 hr at RT. Strips were washed 5x in PBS/0.2% Tween 20, then developed using 0.15 mg/ml 5-bromo-4-chloro-3-indol phosphate/0.3 mg/ml nitro blue tetrazolium in 100 mM NaCl/5 mM $MgCl_2$/100 mM Tris pH 9.5. Development reactions were stopped by placing strips in distilled water. Strips containing molecular weight standards (Mark 12, Novex) were stained 1 min with Coomassie blue then destained in distilled water containing 45% methanol and 4.5% acetic acid.

Immunoprecipitation. 804G cells between passage p20-p30 were split into a medium containing 10% DMEM/90% Eagle's minimal essential medium lacking methionine, cysteine, and glutamine (Sigma), 10% dialyzed fetal calf serum (Biowhittaker), 1% glutamine/penicillin/streptomycin (Irvine Scientific, Santa Ana CA) and labeled with 50 μCi per ml of tran^{35}S-label (ICN). After 48 h the 804G conditioned medium was centrifuged 10 min at 3,000 x g. Immunoprecipitations of cleared supernatant were carried out with 500 μl

conditioned medium per reaction diluted 1:1 with 2 X radioimmunoprecipitation (RIPA) buffer (2% Nonidet P-40, 1% sodium deoxycholate, 0.2% SDS, 300 mM NaCl, 100 mM Tris pH 7.5) and 2 µl of polyclonal serum plus 5 µl anti-rabbit-IgG-agarose beads (Sigma), or 2 µl of purified monoclonal antibody plus 5 µl anti-mouse-IgG agarose beads (Sigma), respectively. Reactions were incubated for 2 h at 4°C, extensively washed in 1X RIPA buffer and eluted by boiling 5 min in Laemmli sample buffer containing β-mercaptoethanol. Samples were then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 6% polyacrylamide gels, which were subsequently soaked 15 min in En^{3}Hance (DuPont), dried and exposed to X ray film (Dupont/NEN, Boston MA). Labeled proteins bands were visualized on developed X ray film.

ELISA assays.: For passively adsorbed laminin-5r plates, 804G cell conditioned medium was diluted 1:4 in DMEM/100mM HEPES pH 7.4 and plated (100 µl/well) in 96 well ELISA plates (Sarstedt, Newton NC). As a control, plates were also coated with unconditioned 804G medium at the same dilution. Plates were stored overnight at 4°C, then washed 2 x with PBS (200 µl/well). For assays using normalized polyclonal reactivity, one set of 8 wells was treated for 5 min with 1 M acetic acid, then washed twice with PBS. For chemical denaturation assays, wells were treated for 5 min with 1 M KOH or 8 M urea, followed by two washes with PBS.

For cell-associated laminin-5r plates, 804G cells (25,000/well) were cultured in 96 well tissue culture plates (Corning, Cambridge MA). FGmet2 cells were grown in control plates. After 2 days, plates were washed 2x with PBS and cells removed by adding 20mM NH_4OH for 5 min, followed by 2 washes with PBS. One set of 8 wells was treated for 5 min with 1 M acetic acid, followed by two washed with PBS.

Passively adsorbed plates were blocked for at least 2 hr with blotto. Cell associated plates were blocked with 3% bovine serum albumin (BSA)/PBST. Primary antibodies were diluted 1:2000 in appropriate blocking buffer and 50 µl added to each well. After 1 hr, plates were washed 2 x with PBST for passively adsorbed and 1%BSA/PBST for cell-associated plates. Goat (anti-mouse or anti-rabbit) secondary antibodies conjugated to horseradish peroxidase (Southern Biotechnology Associates, Inc., Birmingham, AL) diluted 1:2000 in appropriate blocking buffer were added to each well. After 1 hr, wells were washed 4 x with PBST then 2x with PBS. Wells were developed with 100 µl/well o-phenylenediamine (3 µg/ml; Sigma) and H_2O_2 (0.1%; Sigma) in 50mM citrate/50mM Na_2HPO_4 pH 5.0. Development reaction was stopped with 4N H_2SO_4. Plates were read at 490 nm using a Vmax plate reader (Molecular Devices, Menlo Park CA). Background values averaged from

8 wells/antibody on control plates were subtracted from those on experimental plates.

Cell contact. 96 well tissue culture plates coated with passively adsorbed 804G conditioned medium as described above were washed 2 x with PBS. SCC12 cells (25,000/well) were added to these plates as well as control, uncoated plates. At indicated times, plates were processed as for cell-associated ELISA assays. One set of 8 wells on each plate was treated for 5 min with 1 M acetic acid to denature laminin-5r; these wells were incubated with 0668B polyclonal antibody. Background values averaged from 8 wells/antibody on control plates were subtracted. For each time point, monoclonal values were expressed as a percentage of the corresponding polyclonal average for acid-treated wells.

Ammonium sulfate precipitation of soluble laminin 5r. Soluble laminin 5r was collected by ammonium sulfate precipitation of serum-free 804G conditioned medium. Solid ammonium sulfate was added to conditioned medium to a final concentration of 30% saturation and stirred overnight at 4°C. Precipitated material was removed from the supernatant by centrifugation at 10,000 x g for 30 min. Solid ammonium sulfate was added to 40% saturation and the solution was stirred overnight at 4°C. Precipitated laminin 5r was collected by centrifugation at 10,000 x g for 30 min, dissolved and dialyzed in PBS . Fibronectin was removed by mixing with gelatin sepharose (Pharmacia, Alameda CA) for 30 min at room temperature. The supernatant was then concentrated using an Amicon stirred cell (Amicon, Beverly MA) and a 100,000 molecular weight filter. Protein concentration was determined using the microBCA assay (Pierce, Rockford IL).

Monoclonal antibody production. Female Balb/cByJ (6-8 weeks old) were given intraperitoneal injections of 100 µg of ammonium sulfate-precipitated laminin-5r dissolved in 0.5 ml RIBI adjuvant (RIBI ImmunoChem Research, Inc., Hamilton MT). This injection was repeated two and four weeks later. Mouse sera were then collected from tail veins and screened by cell-associated ELISA assay. Positive mice were boosted for three consecutive days with intraperitoneal injections of 100 µg of ammonium sulfate-precipitated laminin-5r dissolved in PBS. The next day, mice were sacrificed by CO_2 asphyxiation and the splenocytes were fused to SP2/0 mouse myeloma cells using established methods. Hybridomas were selected using HAT medium (Sigma) and wells were screened by cell-associated ELISA assay. Positive wells were expanded and cloned twice in succession by sorting single cells into 96 well plates using a Becton-Dickinson FACStar fluorescence activated cell sorter (Becton-Dickinson, Bedford MA) equipped with an Automated Cell Deposition Unit. Single cells were sorted on the basis of side and forward light scatter. Clones were screened by cell-associated ELISA assay; all secondary clones screened were positive. Specificity of monoclonal antibodies for laminin-5r was determined by ELISA assay of

immunodepleted 804G conditioned medium (not shown). Isotypes of antibodies were determined using isotype-specific secondary antibodies (Southern Biotechnology Associates, Inc.) in a cell-associated ELISA assay; 5C5, BH5, CM6, DF2, FM3, and TR1. CM6, FM3 and TR1 were determined to be mouse isotype IgG_1, DF2 is mouse isotype IgG_{2a}, and BH5 is mouse isotype IgG_{2b}. Ascites were induced in Balb/cByJ mice and ascites fluid collected as previously described (Harlow and Lane, 1988).

Polyclonal antibody production. The polyclonal antibody J18 has been described previously (Langhofer et al., 1993). For production of the 0668B polyclonal antibody, 804G cells were directly grown on nitrocellulose (Biorad) until confluent. Cells were removed according to Gospodarowicz (Gospodarowicz, 1984). For the first immunization a 2 x 2 cm piece of nitrocellulose was subcutaneously implanted into male New Zealand white rabbits (6-8 weeks old). Booster injections with cell-associated 804G matrix (20 μg per immunization) were subsequently given four and eight weeks later. Blood was collected from ear veins, incubated for 1 h at 37°C, then stored overnight at 4°C. Following centrifugation for 30 min at 3,000 x g to remove cells, the serum was divided into aliquots and stored at -70°C.

Monoclonal antibody purification. Ascites fluid was delipidated by mixing with dextran sulfate (average molecular weight 8,000; Sigma) and precipitation with 0.5M $CaCl_2$. Delipidated ascites fluid was passed over immobilized protein A (Macroprep; Biorad) and antibodies were eluted with 50 mM sodium citrate/100 mM NaCl pH 6.0. Eluted fractions were immediately exchanged into PBS using a G25 sephadex column (Pharmacia).

Affinity purification of laminin-5r. Monoclonal antibody TR1 was coupled to N-hydroxysuccinimide activated agarose (Affigel, Biorad) following manufacturer's directions, and the resulting affinity matrix was packed into a column. Conditioned medium from 804G cells was passed over the column, then the column was washed with 100 mM NaCl/50 mM sodium phosphate, pH 7.7. Laminin-5r was eluted in 50 mM sodium phosphate/100 mM NaCl, 50 mM diethylamine, pH 11.6. Eluted fractions were immediately neutralized in an equal volume of 100 mM citrate buffer, pH 5.03. Fractions were analyzed by western blot, cell adhesion assay, and SDS-PAGE. Fractions containing laminin-5r were pooled, dialyzed against wash buffer, then aliqoted and frozen at -70°C.

Immunofluorescence microscopy. Fresh tissue from Sprague-Dawley rats was snap-frozen in liquid nitrogen and 8-20 μm sections were cut using a microtome. Frozen sections were fixed in acetone or formaldehyde and processed for immunofluorescence microscopy as described previously (Klatte et al., 1989). Briefly, sections were stained with monoclonal antibodies followed by goat anti-mouse IgG antibodies conjugated to fluorescein isothiocyanate (Southern Biotechnology

Associates, Inc.). Fluorescent images were visualized and photographed using a Zeiss Photomicroscope III (Zeiss, Thornwood NY) or with a Zeiss Axiovert microscope eqipped with a laser scanning confocal attachment (MRC-1024, BioRad, Cambridge MA); Color composite images from the Axiovert were generated using Adobe Photoshop 3.0 (Adobe Systems, Inc.) running on a Quadra 950 computer (Apple, Inc.) and printed using a Tektronix Phaser II-SDX.

Adhesion assays. Non-tissue culture-treated 96 well plates (Sarstedt) were coated with 804G cell-deposited matrix (see above), 804G cell conditioned medium, purified MOPC 31c mouse monoclonal antibody (Sigma), purified fibronectin (50 μg/ml), purified laminin-1 (50 μg/ml), purified vitronectin (50 μg/ml) or indicated concentrations of anti-laminin-5r monoclonal antibodies in 100 mM carbonate buffer, pH 9.3. After two washes with PBST (PBS/0.2% Tween 20), wells were blocked with blotto (5% nonfat dried milk/0.2% Tween 20/0.01% thimerosal in PBS). After two washes with PBST, 804G conditioned medium was added to antibody-containing wells, thereby allowing for "capture" of soluble laminin-5r. Wells were washed twice more with PBST.

For antibody blocking experiments, wells were incubated with indicated concentrations of blocking antibodies diluted in blotto. As controls, wells were blocked with blotto alone or irrelevant antibody MOPC 31c or anti-laminin-5r monoclonal antibody FM3. Wells were washed twice with PBST.

Suspended cells were added to wells (8 x 10^4 cells/well) in DMEM/1%BSA/25mM HEPES pH 7.4 and incubated at 37°C/5% CO_2 for 30 minutes. To remove unbound cells, the plates were inverted in a tank of PBS and gently shaken for 15 minutes. Bound cells were fixed in 3% paraformaldehyde/PBS, then stained with 0.5% crystal violet in 20% methanol/80% H_2O. Wells were washed with water to remove excess dye, then cells were solubilized in 1% SDS and amount of dye was quantitated using a Molecular Devices plate reader set at 595 nm.

Results

1. Monoclonal antibodies against laminin-5r.

As stated in the original proposal, the first Specific Aim of this project was to isolate cell surface complexes associated with laminin-binding integrins (α2β1, α6β1) from normal human mammary epithelial cells and from three human mammary cell lines that display varying degrees of metastatic capability. Implicit in this aim was that a well characterized and commercially available form of laminin, originally known as EHS laminin and now known as laminin-1(Burgeson et al., 1994), would be used as the adhesive ligand for inducing integrin complexes in mammary epithelial cells. However, since the submission of the original grant, the focus of this laboratory has shifted away from laminin-1 and towards a

newer, less-well characterized isoform of laminin, which resembles laminin-5 and is derived from a rat cell line. We thus call this laminin isoform laminin-5r (Langhofer et al., 1993)[2].

The reason for this shift in focus is that human and rat forms of laminin-5 are likely to be more physiologically relevant ligands for epithelial cells when compared to laminin-1. For example, hemidesmosomes play a critical role in linking epithelial sheets to the underlying basement membrane, yet epithelial cells traditionally form few and poorly formed hemidesmosomes in vitro when cultured on standard ECM molecules (e.g., fibronectin, laminin-1, vitronectin, collagen type I). In contrast, when cultured on laminin-5-containing matrix, epithelial cells adhere and spread rapidly and form mature hemidesmosomes (Langhofer et al., 1993)[2].

Because of this shift in focus, the objectives of the first specific aim have been modified, and the work during the first year has focused exclusively on this revised first specific aim. Our objectives have been to isolate and characterize laminin-5r sufficiently that it may be used as a ligand for inducing formation of known integrin complexes. Compared to laminin-1, laminin-5 is a poorly characterized molecule. Neither human nor rat laminin-5 is available commercially. Consequently, our goals included the development of reagents that would facilitate isolation and characterization of laminin-5r.

We raised a panel of monoclonal antibodies against laminin-5r and tested these antibodies in ELISA, immunoprecipitation and western blotting assays. After screening fusion wells by ELISA assay on 804G cell deposited matrix, positive hybridomas were expanded and cloned. Those hybridomas that reacted positively by ELISA assay after secondary subcloning were further characterized by western blot (Fig. 2) and immunoprecipitation (Fig. 3). We isolated five hybridomas (BH5, CM6, DF2, FM3, TR1) that successfully recognized laminin-5r in these assays. Western blots and immunoprecipitations performed using reducing conditions in SDS-PAGE resolve laminin-5r into three major bands of apparent molecular weight 150, 140, and 135 kDa and two minor bands of apparent molecular weight 100 and 80 kDa. Protein sequencing by Dr. Jutta Falk-Marzillier in our laboratory has identified each of these bands as containing the constituent chains of laminin-5r (Fig. 4). Each of the monoclonal antibodies isolated recognized at least one of these bands by western blot and successfully immunoprecipitated the entire laminin-5r complex from 804G cell conditioned medium. None of the monoclonal antibodies tested crossreacted with human laminin-5 in ELISA assay (not shown) or immunoprecipitation (Fig. 3), suggesting that they are specific for laminin-5r.

2. Affinity purification of laminin-5r.

We used one these monoclonal antibodies to affinity purify laminin-5r

from conditioned media of 804G cells (Fig. 5). TR1 coupled to N-hydroxysuccinimide activated agarose was used to capture laminin-5r from 804G conditioned medium, and bound laminin-5r was eluted in a high pH buffer. Eluted protein was separated by SDS-PAGE, and resolved into five bands representing laminin-5r, as expected from the protein sequencing data.

We originally intended to use affinity purification as a means of obtaining large quantities of purified laminin-5r. However, we discovered during purification that as laminin-5r becomes increasingly enriched (i.e., during washing and elution of the affinity column), it spontaneously becomes insoluble and consequently clogs the affinity column, leading to a large and potentially harmful backpressure in our chromatography system. We are currently attempting to purify laminin-5r using more conventional biochemical methods (e.g., ion exchange and gel filtration chromatography).

3. Use of monoclonal antibodies to examine the structure of laminin-5r.

How laminin-5r is assembled into basement membranes is unknown. Current models suggest that laminin heterotrimers self assemble in a Ca^{+2}-dependent manner to form an insoluble matrix within the basement membrane of epithelial cells. The amino terminal globular domains of the short arms are thought to play critical roles in the polymerization of laminin heterotrimers (Yurchenco and Cheng, 1993; Schittny and Schittny, 1993). Laminin-5r is closely related to human laminin 5 (Jones et al., submitted) and thus is distinguished from "classical laminin" (EHS laminin, laminin-1) in that it lacks these amino terminal globular "assembly" domains in the α and γ chains (Ryan et al., 1994; Kallunki et al., 1992). It is therefore likely that the mechanism of laminin-5r assembly differs from that for laminin 1.

Laminin-5r exists in three physical forms in vitro: freely soluble in aqueous solution (e.g., cell culture media), passively adsorbed to surfaces (e.g., cell culture plastic), and cell-associated (e.g., beneath epithelial cell monolayers). In this study we investigated the structure of laminin-5r by using monoclonal antibodies to detect specific epitopes in each of these three forms of laminin-5r. We found that soluble, passively adsorbed, and cell-associated laminin-5r exhibited a distinct set of monoclonal antibody epitopes. Importantly, plating the human epithelial cell line SCC12 on passively adsorbed laminin-5r unveiled epitopes expressed on cell-associated laminin-5r. Together these data suggest that cell contact may "convert" passively adsorbed to cell-associated laminin-5r and thus may play a critical role in assembly of a laminin-5r matrix.

<u>The 5C5 epitope is only exposed in cell-associated laminin-5r.</u>

We have previously described a monoclonal antibody, 5C5, which

recognizes the 150 kDa α_3 chain of laminin-5r (Langhofer et al., 1993). Western blots of soluble and cell-associated laminin-5r revealed the 5C5 epitope in both forms (Fig. 2). However, 5C5 failed to immunoprecipitate the soluble form of laminin-5r from 804G cell conditioned media but did precipitate cell-associated laminin-5r (Fig. 6), suggesting that the 5C5 epitope was inaccessible to antibody in the soluble form of the molecule.

Immunofluorescence microscopy of rat tongue sections demonstrated that the 5C5 epitope was expressed in basement membranes (Fig. 7) , suggesting that this epitope is unveiled in the cell-associated form of laminin-5r in vivo. This observation raised the possibility that soluble and cell-associated forms of laminin-5r are conformationally distinct. This hypothesis was tested by comparing in ELISA assays soluble laminin-5r passively adsorbed to plates vs. cell-associated laminin-5r. The 5C5 epitope was not detectable by ELISA assay on passively adsorbed but was apparent on cell-associated laminin-5r (Fig. 8). Denaturation of passively adsorbed laminin-5r with acid, base, or urea increased polyclonal antibody reactivity but did not induce the appearance of 5C5 epitope (Fig. 9). This suggested that cell contact was responsible for 5C5 epitope appearance on the molecular surface of laminin-5r.

Monoclonal antibodies distinguish between three forms of laminin-5r.

We repeated these assays using five new monoclonal antibodies raised against laminin-5r. We found that four of these antibodies reacted strongly with both soluble and cell-associated laminin-5r in western blots; BH5 reacted weakly on cell-associated and failed to react with soluble laminin-5r (Fig. 6). All reactive antibodies recognized at least one major chain of the laminin-5r complex. Unlike 5C5, all of these antibodies also immunoprecipitated soluble laminin-5r, indicating that each of these epitopes is present on the molecular surface of soluble laminin-5r (Fig 3). While the J18 and 0668B polyclonal antibodies cross reacted with human laminin 5 from SCC25 cell conditioned media (Fig 10, lanes 2 and 4), the monoclonal antibodies did not, demonstrating that these monoclonal antibodies are specific for laminin-5r.

We used ELISA assays to directly compare the monoclonal antibody epitope expression in passively adsorbed and cell-associated forms of laminin-5r. Addition of 1M acetic acid to laminin-5r-coated wells resulted in increased polyclonal antibody signal in ELISA assays relative to untreated wells (Fig. 9) and completely abolished cell adhesion to passively adsorbed laminin-5r (data not shown). We therefore used this treatment to denature laminin-5r and remove any conformation-sensitive epitopes. We normalized the $O.D._{490}$ values obtained for monoclonal antibodies in untreated wells to polyclonal anti-laminin-5r antibodies on acid-treated, denatured wells. A schematic representation of this approach is shown in Fig. 9. We found that like 5C5, two monoclonal antibodies (BH5, DF2) failed to react on passively adsorbed laminin-5r but did react on cell-associated laminin-5r (Fig. 10).

Taken together, these results demonstrated that 1) some epitopes present on the surface of soluble laminin-5r are lost upon adsorption of this molecule to plastic, while others are not, and 2) passively adsorbed and cell-associated laminin-5r express a different complement of monoclonal antibody epitopes. This differential expression of epitopes makes it likely that the three forms of laminin-5r are conformationally distinct.

Cell contact induces conversion of the passively adsorbed form to cell-associated form of laminin-5r.

The above observations raised the possibility that cell contact plays a critical role in determining the conformation of laminin-5r coated on solid substrates. We tested this directly by plating human SCC12 cells on passively adsorbed laminin-5r and monitoring monoclonal antibody epitopes by ELISA assay. Because the 5C5, BH5 and DF2 epitopes were not accessible on passively adsorbed laminin-5r, we chose to focus on these epitopes.

To control for synthesis and/or degradation of laminin-5r by SCC12 cells during the course of cell contact, we monitored the total amount of amount of laminin-5r present using 0668B antibody reactivity in acid-treated wells. At each time point, values obtained from wells containing monoclonal antibodies were calculated as a percentage of the values obtained from acid-treated wells containing polyclonal antibody.

Within three days of cell plating, reactivity of the 5C5, BH5 and DF2 epitopes increased substantially, although these increases occurred at different times (Fig 11). Reactivity for the CM6, FM3, and TR1 antibodies also increased over this time course (not shown). These data suggested that SCC12 cell contact converted laminin-5r from a passively adsorbed to a cell-associated conformation.

In summary, we have used monoclonal antibodies against laminin-5r to demonstrate that soluble, passively adsorbed, and cell-associated forms of laminin-5r express a distinct set of surface epitopes and therefore are conformationally distinct. Importantly, cell contact with passively adsorbed laminin 5r induces unveiling of the 5C5, BH5 and DF2 epitopes. This data is summarized in Table I.

4. Adhesion assays with laminin-5r.

Laminin-5r induces rapid adhesion and spreading of mammary epithelial cells.

The purpose of this study was to determine the morphological effects of binding laminin-5r on normal and malignant mammary epithelium. To do so, we bound normal and malignant human mammary epithelial cells to

laminin-5r. The laminin-5r was presented as 804G cell deposited matrix or 804G conditioned medium passively adsorbed to plastic. Both forms supported adhesion (Fig. 12) and spreading (not shown) of normal mammary epithelial cells within 30 minutes. The malignant cell lines MCF-7, MDA-MB-435, and MDA-MB-231also adhered to laminin-5r during this time course. All four cell types bound well to human laminin-1, and bound poorly to human fibronectin and rat laminin-1.

Definition of an adhesion epitope on laminin-5r using the monoclonal antibody CM6.

Because the adhesion assays described above were conducted with non-purified laminin-5r, the specific contribution of laminin-5r in these preparations could not be discerned. To overcome this difficulty, we adopted a modified adhesion assay wherein plastic wells were coated with highly purified anti-laminin-5r monoclonal antibodies, which act as "capturing agents" for soluble laminin-5r. As a result, we were able to affinity purify laminin-5r within individual wells of a 96-well plate. These plates were then used in rapid adhesion assays.

Normal and malignant mammary epithelial cells attached to and spread on affinity captured laminin-5r (Fig. 13), consistent with the data obtained with non-purified laminin-5r. Interestingly, we found that capturing of laminin-5r with the monoclonal antibody CM6 completely eliminated adhesion of all four cell types. As determined by ELISA assay, CM6 is a slightly more efficient capturing agent than is TR1 (data not shown), suggesting that adhesion wells coated with CM6 contained at least as much laminin-5r as wells coated with TR1. We also observed that addition of CM6 to wells containing laminin-5r captured by TR1 blocked adhesion of a number of epithelial cell types by approximately 90% (e.g., SCC12 cells, Fig. 14). Finally, we observed that addition of CM6 to cultures of 804G cells caused the cells to round up and detach from their matrix (not shown). Taken together these findings suggest that CM6 recognizes an epitope on laminin-5r that is also recognized by epithelial cell adhesion receptors, and that CM6 effectively competes for this site and thereby blocks cell adhesion.

In summary, we have demonstrated that laminin-5r is a physiologically relevant adhesion ligand for mammary epithelial cells. Second, laminin-5r assumes at least three structural conformations as defined by expression of monoclonal antibody epitopes, and cell contact plays an important role in establishment of the most stable of these forms, which we call cell-associated. Finally, we have identified a major cell binding site on laminin-5r using a monoclonal antibody. The implications of these findings and their impact on the project outlined in this grant are discussed below.

Conclusions

One of the most important goals of this project is to develop an appropriate model system to study interactions of mammary epithelial cells with defined, physiologically relevant components of their basement membrane. We have chosen to define this relevance in terms of how well a given matrix molecule supports formation of stable integrin-mediated adhesion complexes in normal epithelial cells. Published reports suggest that laminin-5 is more efficient at promoting formation of epithelial-specific adhesion complexes (hemidesmosomes) than the more well-characterized laminin isoform, laminin-1. As a result, our first specific aim has been modified to replace laminin-1 with laminin-5r in our system. Because laminin-5 is a relatively new molecule, considerable effort during the first year of this project has been devoted to isolating and characterizing laminin-5r for its inclusion in our model system.

The data thus far obtained are highly encouraging. We conclude that laminin-5r is a physiologically relevant ligand for mammary epithelial cells because it is present in the basement membrane of rat mammary epithelium, and, more importantly, it promotes rapid adhesion and spreading of normal mammary epithelial cells. We have also observed that laminin-5r promotes formation of hemidesmosomes in human epithelial cells in vitro. We have yet to establish that mammary epithelial cells form hemidesmosomes when plated on laminin-5r; nonetheless, the data suggest that mammary epithelial cell interactions with laminin-5r are physiologically relevant.

A second, crucial requirement of our model system is that it should allow us to functionally distinguish between normal and malignant phenotypes in mammary epithelium. We observe that malignant mammary epithelial cells bind less well to cell associated laminin-5r, and at least one cell line, MCF7, binds more strongly to soluble and passively adsorbed laminin-5r when compared to normal mammary cells. These observations suggest that we have met this second requirement and are therefore justified in utilizing laminin-5r in our model.

Use of such a model system allows us to develop immediately testable hypotheses. Our goals for the second year of this grant are to test the following hypotheses:

1. Changes in adhesion and spreading on laminin-5r in malignant cells is due to modified expression of integrin receptors for laminin-5r. Our first goal will be to determine which known integrins are responsible for adhesion to laminin-5r. Whereas the integrin receptors for laminin-1 are well known and characterized, relatively little is known about the integrins responsible for binding laminin-5r. Immunofluorescence microscopy has revealed that the hemidesmosome-associated integrin $\alpha 6\beta 4$ codistributes with laminin-5r, suggesting that $\alpha 6\beta 4$ may bind laminin-5r (Langhofer et al., 1993; Marchisio et al., 1993; Sonnenberg et

al., 1993). Inhibitory antibodies against α6, β1, and α3 have also decreased epithelial cell adhesion to laminin-5-containing matrices, thereby implicating α3β1, α6β1, and α6β4 as potential receptors for laminin-5r (Wayner et al., 1993; Verrando et al., 1994; Niessen et al., 1994). We will make use of a number of cell types that adhere well to laminin-5r (e.g., SCC12, FGmet2) and commercially available antibodies that block function of known laminin-binding integrins (e.g., α6, α3, β1) in our affinity capture adhesion assay to determine which integrins are responsible for adhesion to laminin-5r. Once we have identified candidate receptors for laminin-5r, we can probe normal and transformed mammary epithelial cells by western blot, immunoprecipitation, and/or fluorescence activated cell sorting (FACS) to determine the level of expression of these integrins in our cells. Interestingly, it has been previously determined in this lab that all three malignant mammary cell lines we are using express relatively low levels of both α6 and β4 integrins; the MDA-MB-435 cell line does not express β4 at all (data not published). Lowered expression of these integrins may correlate with the relatively poor adhesion to cell-associated laminin-5r.

2. Laminin-5r contains at least one adhesion site used by mammary epithelial cells; both normal and transformed cells use the same adhesion site(s). Because our monoclonal antibody CM6 eliminates adhesion of all cell types to laminin-5r, we expect to find at least one cell binding domain within the epitope recognized by CM6. Our plan is to test defined fragments of laminin-5r for CM6 recognition by ELISA assay; those fragments that contain the CM6 epitope will be further screened by adhesion assay. Adhesion activity of these fragments will be confirmed by subsequent blocking with CM6 antibody in the adhesion assay. To achieve this goal, we will work in close cooperation with other members of this lab who will be cloning and expressing the constituent chains of laminin-5r in the coming year. We hope that they can provide us with defined fragments of these chains and that by examining progressively smaller fragments we will be able to map the adhesion site with a great deal of precision. Our expectation is that both normal and transformed cells will recognize the same site on laminin-5r, but that transformed cells will bind less strongly to this site, as defined by fewer cells binding during adhesion assays.

3. Adhesion to laminin-5r via specific integrin receptors activates biochemical signalling pathways that are responsible for maintaining the normal phenotype; malignant cells fail to activate these pathways. Having established which integrins are responsible for adhesion to laminin-5r and the binding site for these integrins on laminin-5r, we hope to identify biochemical signalling pathways that are activated by clustering of these integrins upon adhesion to laminin-5r. To do so, we will employ the magnetic microbead isolation technique described in the original grant which has been used to characterize the chemical

signaling activity associated with integrin complexes[3], using laminin-5r as the integrin ligand. Until we develop a more efficient means of obtaining purified laminin-5r, we will use beads coated with anti-laminin-5r antibodies to affinity capture laminin-5r from 804G cell conditioned media. These beads will be used to induce clustering of laminin-5r receptors, followed by formation of integrin-associated adhesion complexes. Isolation and biochemical characterization of these complexes will be accomplished using the approaches described in the original grant. We anticipate that malignant cells will fail to activate chemical signalling pathways accompanying integrin-specific binding to laminin-5r.

[3]Plopper,G.E., H.P. McNamee, L.E. Dike, K. Bojanowski, and D.E. Ingber. (1995) Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. Mol. Biol. Cell (in press).

References.

Burgeson, R.E., Chiquet, M., Deutzmann, R., Ekblom, P., Engel, J., Kleinman, H., Martin, G.R., Meneguzzi, G., Paulsson, M., Sanes, J., Timpl, R., Tryggvason, K., Yamada, Y., and Yurchenco, P.D. (1994). A new nomenclature for the laminins. Matrix Biol. *14*, 209-211.

Gospodarowicz, D. (1984). Methods for Preparation of Media, Supplements and Substrata (New York: Alan R. Liss, Inc.).

Harlow, E. and Lane, D. (1988). Antibodies: A Laboratory Manual (Cold Spring Harbor: Cold Spring Harbor Laboratory).

Hynes, R.O. (1992). Integrins: Versatility, modulation and signaling in cell adhesion. Cell *69*, 11-25.

Kallunki, P., Sainio, K., Eddy, R., Byers, M., Kallunki, T., Beck, K., Hirvonen, H., Shows, T.B., and Tryggvason, K. (1992). A truncated laminin chain homologous to the B2 chain: structure, spatial expression, and chromosomal assignment. J. Cell Biol. *119*, 679-693.

Kibbey, M.C., Yamamura, K., Jun, S.H., Grant, D.S., and Kleinman, H.K. (1994). Enhancement of tumor growth by basement membrane: modulation of. Cancer Treat Res *71*, 267-275.

Klatte, D.H., Kurpakus, M.A., Grelling, K.A., and Jones, J.C. (1989). Immunochemical characterization of three components of the. J. Cell. Biol. *109*, 3377-3390.

Langhofer, M., Hopkinson, S.B., and Jones, J.C.R. (1993). The matrix secreted by 804G cells contains laminin related components that participate in hemidesmosome assembly in vitro. J. Cell Sci. *105*, 753-764.

Marchisio, P.C., Cremona, O., Savoia, P., Pellegrini, G., Verrando, P., Burgeson, R.E., Cancedda, R., and De Luca, M. (1993). The basement membrane protein BM-600/nicein codistributes with kalinin and the integrin alpha 6 beta 4 in human cultured keratinocytes. Exp. Cell Res. *205*, 205-212.

Niessen, C.M., Hogervorst, F., Jaspars, L.H., de Melker, A.A., Delwel, G.O., Kuikman, I., and Sonnenberg, A. (1994). The alpha 6 beta 4 integrin is a receptor for both laminin and kalinin. Experimental Cell Research *211*, 360-367.

Rousselle, P. and Aumailley, M. (1994). Kalinin is more efficient than laminin in promoting adhesion of primary keratinocytes and some other epithelial cells and has a different requirement for integrin receptors. J. Cell. Biol. *125*, 205-214.

Ryan, M.C., Tizard, R., VanDevanter, D.R., and Carter, W.G. (1994). Cloning of the LamA3 gene encoding the alpha 3 chain of the adhesive ligand epiligrin. Expression in wound repair. Journal of Biological Chemistry *269*, 22779-22787.

Schittny, J.C. and Schittny, C.M. (1993). Role of the B1 short arm in laminin self-assembly. European Journal of Biochemistry *216*, 437-441.

Sonnenberg, A. (1993). Integrins and their ligands. [Review]. Current Topics in Microbiology & Immunology *184*, 7-35.

Sonnenberg, A., de Melker, A.A., Martinez de Velasco, A.M., Janssen, H., CalafatJ., , and Niessen, C.M. (1993). Formation of hemidesmosomes in cells of a transformed murine mammary tumor cell line and mechanisms involved in adherence of these cells to laminin and kalinin. J. Cell. Sci. *106*, 1083-1102.

Sweeney, T.M., Kibbey, M.C., Zain, M., Fridman, R., and Kleinman, H.K. (1991). Basement membrane and the SIKVAV laminin-derived peptide promote. Cancer Metastasis Rev *10*, 245-254.

Tryggvason, K. (1993). The laminin family. [Review]. Current Opinion in Cell Biology *5*, 877-882.

Verrando, P., Lissitzky, J.C., Sarret, Y., Winberg, J.O., Gedde-Dahl, T., Schmitt, D., and Bruckner-Tuderman, L. (1994). Keratinocytes from junctional epidermolysis bullosa do adhere and migrate on the basement membrane nicein through alpha3beta1 integrin. Laboratory Investigation *71*, 567-574.

Wayner, E.A., Gil, S.G., Murphy, G.F., Wilke, M.S., and Carter, W.G. (1993). Epiligrin, a component of epithelial basement membranes, is an adhesive ligand for alpha 3 beta 1 positive T lymphocytes. J. Cell Biol. *121*, 1141-1152.

Yurchenco, P.D and Cheng, Y.-S. (1994). Laminin self-assembly: A three-arm interaction hypothesis for the formation of a network in basement membranes. Contrib. Nephrol. *107*, 47-56.

Yurchenco, P.D. and Cheng, Y.-S. (1993). Self-assembly and calcium binding sites in laminin: A three-arm interaction model. Journal of Biological Chemistry *268*, 17286-17299.

Appendix.

Figure legends.

Figure 1. Confocal immunolocalization of laminin-5r in rat mammary gland. Adult female rat tissue was snap frozen in liquid nitrogen, sectioned, fixed with paraformaldehyde, and subsequently stained with monoclonal antibodies BH5 (A, B) or CM6 (C, D) against laminin-5r. Note intense staining of basement membrane surrounding epithelial cells lining mammary ducts. Magnification = 40X.

Figure 2. Western blot of soluble (A) and cell-associated (B) laminin-5r with polyclonal antibodies (J18, 0668B) and the indicated monoclonal antibodies. Note that each monoclonal antibody recognizes at least one major band in the 150-130 kD range, indicative of the intact laminin-5r chains. DF2, FM3, and TR1 all recognize bands of approximately 80 kD in cell-associated laminin-5r. It is likely that these represent proteolytic products of laminin-5r. Migration of molecular weight standards is indicated at left.

Figure 3. New monoclonal antibodies immunoprecipitate soluble laminin-5r. Indicated antibodies were added to ^{35}S-labeled conditioned media from rat 804G (odd numbered lanes) or human SCC25 cells (even numbered lanes) and resulting immunoprecipitations were separated by SDS-PAGE and analyzed by fluorography. Note that the polyclonal antibodies J18 and 0668B cross-react with human laminin 5 but that the monoclonal antibodies do not. Note also that preimmune serum from rabbit 0668 (pi) fails to precipitate laminin-5r or human laminin 5. Migration of molecular weight markers are indicated at left.

Figure 4. N-terminal and internal amino acid sequencing of 804G cell matrix components. All analyzed polypeptides show sequence homology to human laminin-5 α, β, and γ chains, suggesting that the laminin isoform in 804G matrix represents a rat homologue. Note that the α3 and γ2 chains exist in several forms.

Figure 5. Affinity purification of laminin-5r. 804G cell conditioned medium was passed over a column of bound TR1 monoclonal antibody, and bound products were eluted to high pH buffer. Eluted proteins were concentrated using a centricon filter, separated by SDS-PAGE, and stained with coomassie blue. Migration of molecular weight standards are indicated at left, calculated molecular weights of eluted polypeptides are indicated at right.

Figure 6. Immunoprecipitation of laminin-5r from 804G conditioned medium with the polyclonal antibody 0668B (lane 1) and the monoclonal antibody 5C5 (lane 2). As controls, 804G conditioned medium (lane 3) and cell-associated laminin-5r (lane 4) were solubilized in 0.1%SDS/100

mM β-mercaptoethanol, dialyzed in PBS, and laminin-5r was precipitated with the 5C5 antibody. Immunoprecipitates were separated by SDS-PAGE and analyzed by fluorography. Migration of molecular weight standards is indicated at left. Note that 5C5 precipitated only cell-associated laminin-5r.

Figure 7. Laminin-5r monoclonal antibody epitopes are expressed in vivo. Rat tongue sections were stained with 5C5, BH5, and CM6 antibodies followed by fluorescein-conjugated secondary antibodies. Staining with secondary antibody alone was used as a control. The photos are aligned so that the epidermis is located at the top. Note that each monoclonal antibody stained the basement membrane intensely.

Figure 8. The 5C5 epitope is expressed only on cell-associated laminin-5r. ELISA assays were performed on passively adsorbed and cell-associated laminin-5r using 5C5 and 0668B antibodies. Note that denaturation of passively adsorbed laminin-5r with 1M acetic acid, 1M KOH, or 8M urea increased polyclonal antibody reactivity but failed to induce 5C5 reactivity. Values shown represent statistical mean +/- SEM (n=8).

Figure 9. Schematic representation of comparative ELISA assay. 96-well plates are coated with 804G conditioned medium or 804G cells. After 2 days, 804G cells are removed with NH_4OH. One set of 8 wells in both plates (bold arrows) is treated with 1M acetic acid to denature laminin-5r, then incubated with the polyclonal antibody 0668B during the ELISA assay. Remaining wells are incubated with monoclonal antibodies. Following development of ELISA assay, optical density values obtained from wells containing polyclonal antibodies are averaged and the ratio of the averages from the two plates is calculated. This ratio is used to normalize the values obtained from the wells treated with monoclonal antibodies on both plates. Data for wells containing monoclonal antibody is expressed as normalized optical density.

Figure 10. Passively adsorbed and cell-associated laminin-5r express distinct monoclonal antibody epitopes. ELISA assays were performed on passively adsorbed and cell-associated laminin-5r. Values expressed represent statistical means +/- SEM (n=8) after normalizing for polyclonal antibody reactivity. Note that BH5 and DF2 epitopes are not expressed on passively adsorbed laminin-5r but that all epitopes are present on cell-associated laminin-5r.

Figure 11. SCC12 cells induce expression of monoclonal epitopes on passively adsorbed laminin-5r. SCC12 cells were cultured on passively adsorbed laminin-5r for the indicated times and then prepared for cell-associated ELISA assays. Results of monoclonal antibody reactivity are expressed as a mean percentage +/- SEM of 0668B antibody reactivity in acid-denatured samples for each time point (n=8).

Figure 12. Mammary epithelial cell adhesion to extracellular matrix proteins (LM-5r, laminin-5r; LM-1, laminin-1; FN, fibronectin; FCS, fetal calf serum). 96-well plates were coated with the indicated matrix proteins and normal (NHMEC) and malignant mammary epithelial cells (MDA-MB-231, MDA-MB-435, MCF7) were allowed to attach for 30 minutes. After washing, adherent cells were fixed, stained with crystal violet, and the amount of dye quantitated by solubilizing in SDS and densitometric scanning. Note that malignant cell lines bound cell associated laminin-5r less well than did normal cells, and that MCF7 cells bound passively adsorbed laminin-5r better than normal cells. Also, none of the cells bound rat laminin-1 or human fibronectin. Results expressed as the statistical mean +/- standard error of the mean (n=4 for all but NHMEC, where n=8).

Figure 13. Mammary cell adhesion to affinity captured laminin-5r. 96 well plates were coated with the indicated anti-laminin-5r monoclonal antibodies, then wells were incubated with 804G cell conditioned medium, thereby "capturing" soluble laminin-5r. As controls, wells were coated with MOPC mouse IgG or blotto alone. After washing, cells were allowed to adhere for 30 minutes. Non-adherent cells were removed by washing and adherent cells were fixed, stained, and quantitated as described for Figure 12. Note that laminin-5r captured by TR1 supports adhesion of each cell type, whereas laminin-5r captured by CM6 does not. Also, MCF7 cells bind captured laminin-5r better than normal cells. Results are expressed as the statistical mean +/- standard deviation (n=8).

Figure 14. Inhibition of cell binding by CM6 monoclonal antibody. Adhesion assay of SCC12 cells plated on 96 well plates "affinity coated" with laminin-5r using anti-laminin-5r monoclonal antibody TR1 as a capturing agent and blocked with the indicated antibodies (all used at 50 μg/ml). As a control, wells were blocked with blotto alone. Results are expressed as the statistical mean +/- standard deviation (n=3).

Figure 1

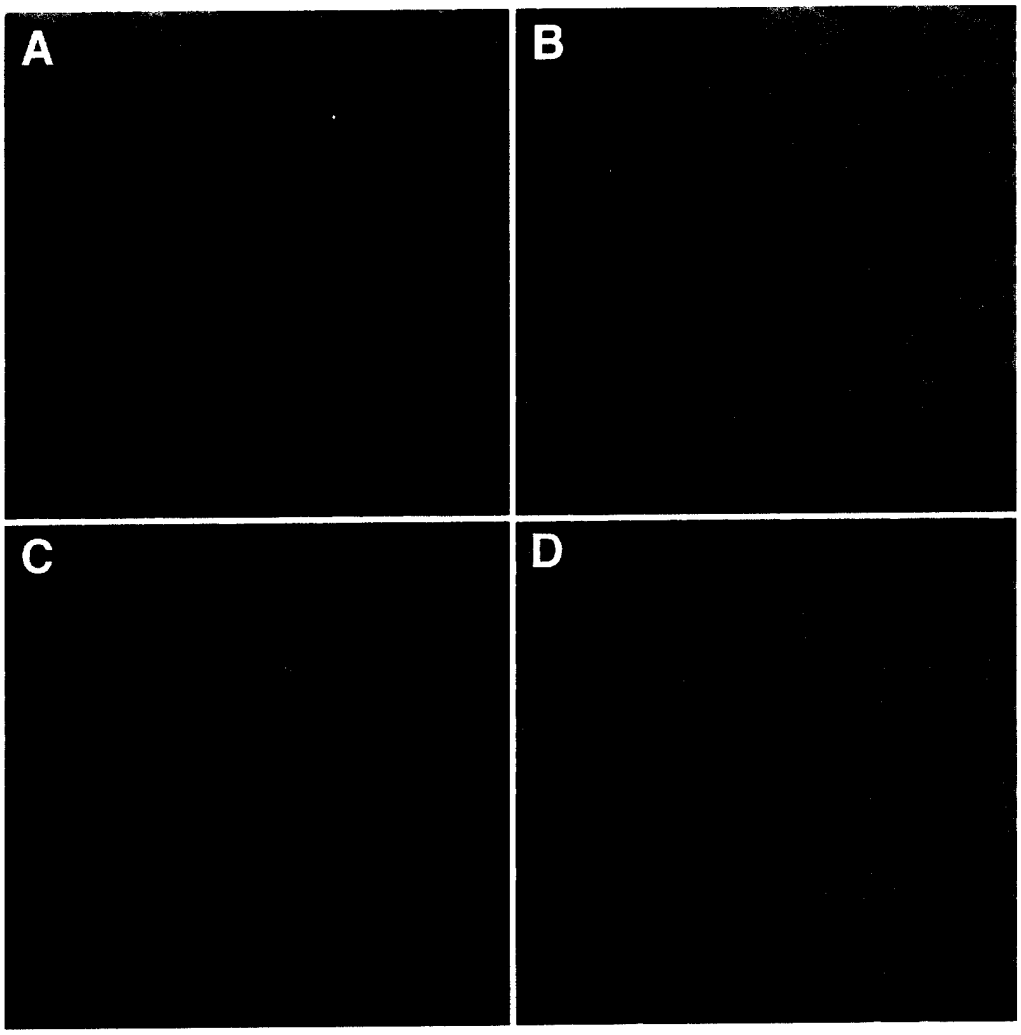
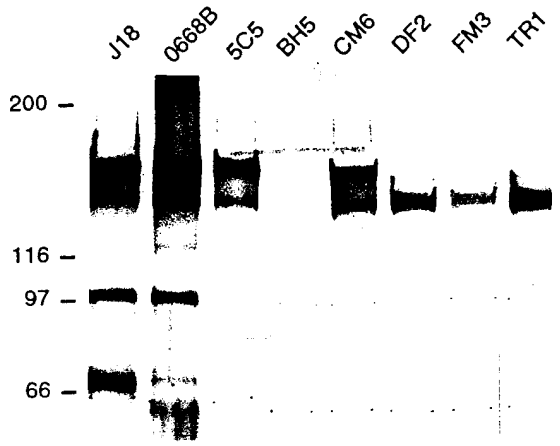


Figure 2

A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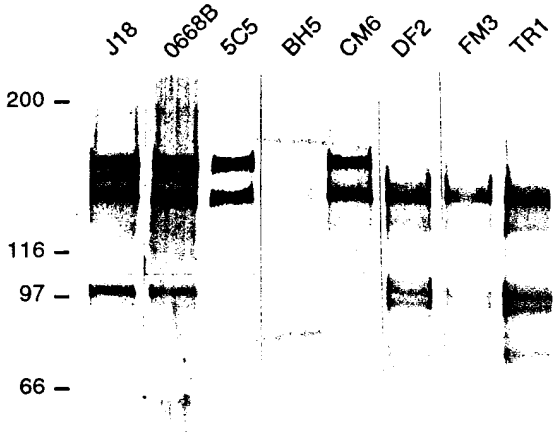
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Figure 3

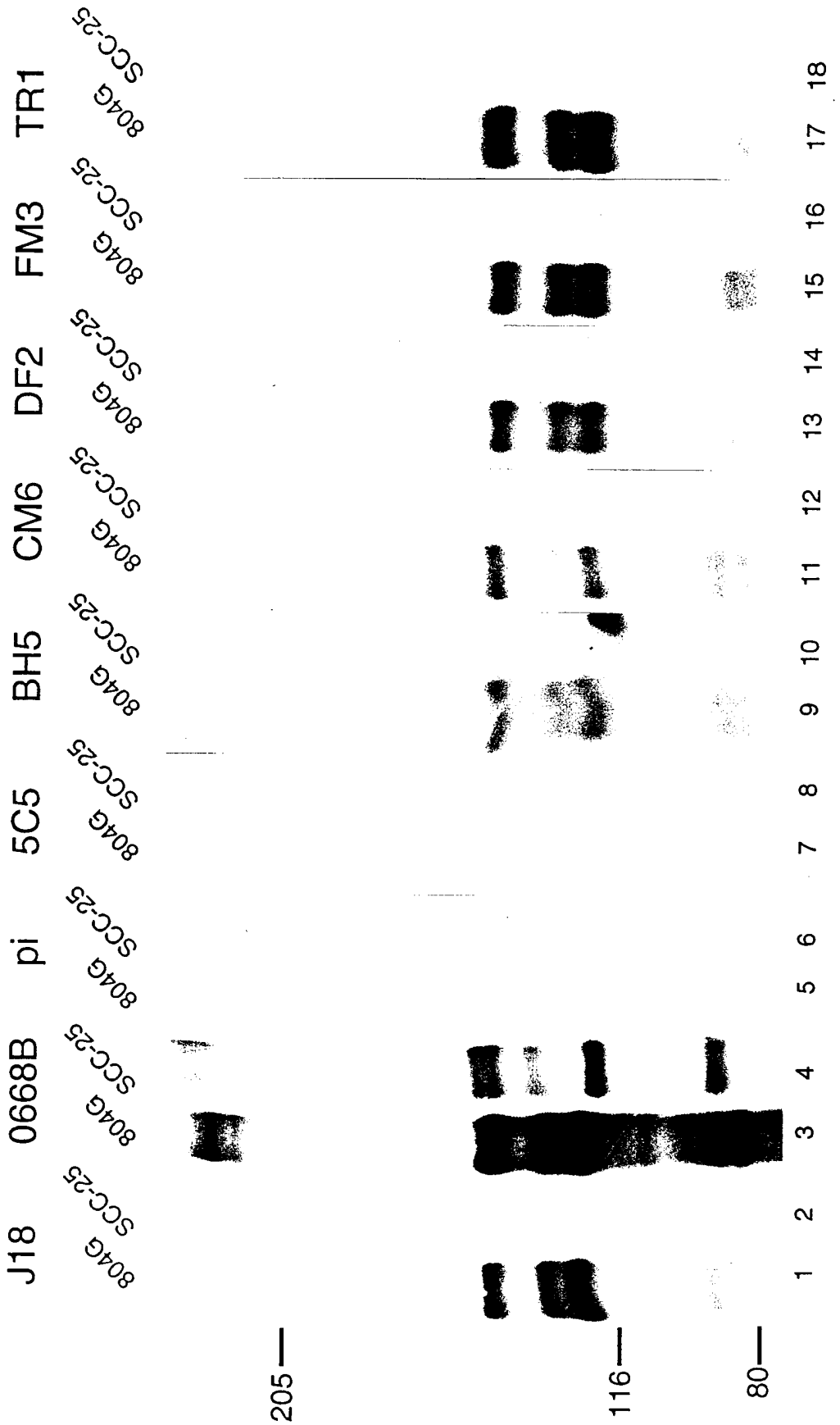
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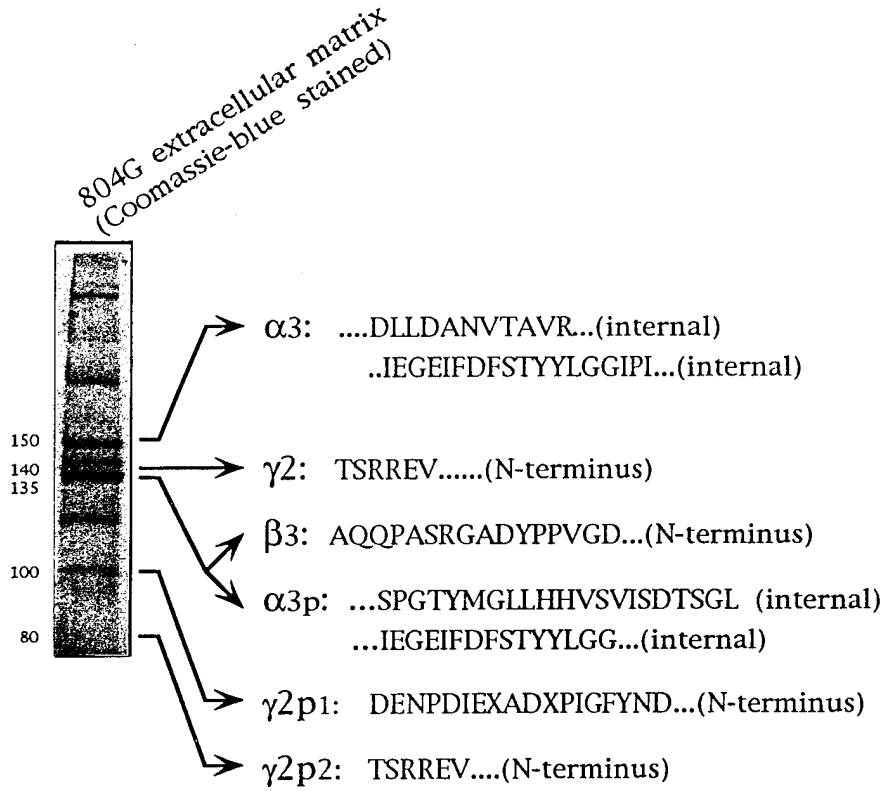
Figure 4

Amino acid sequencing of 804G cell matrix components



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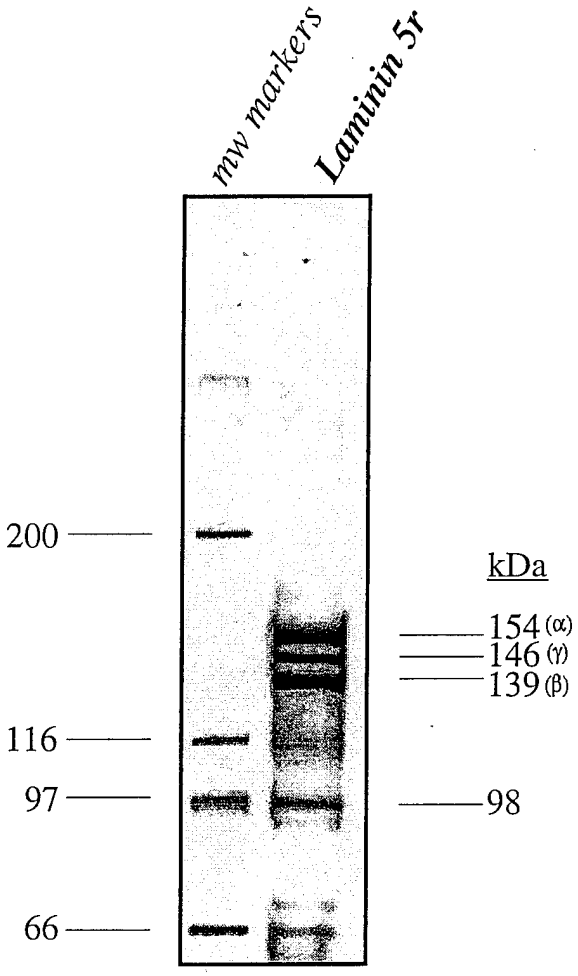

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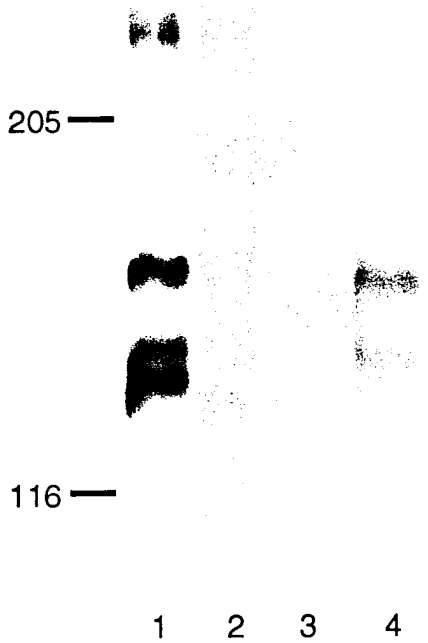

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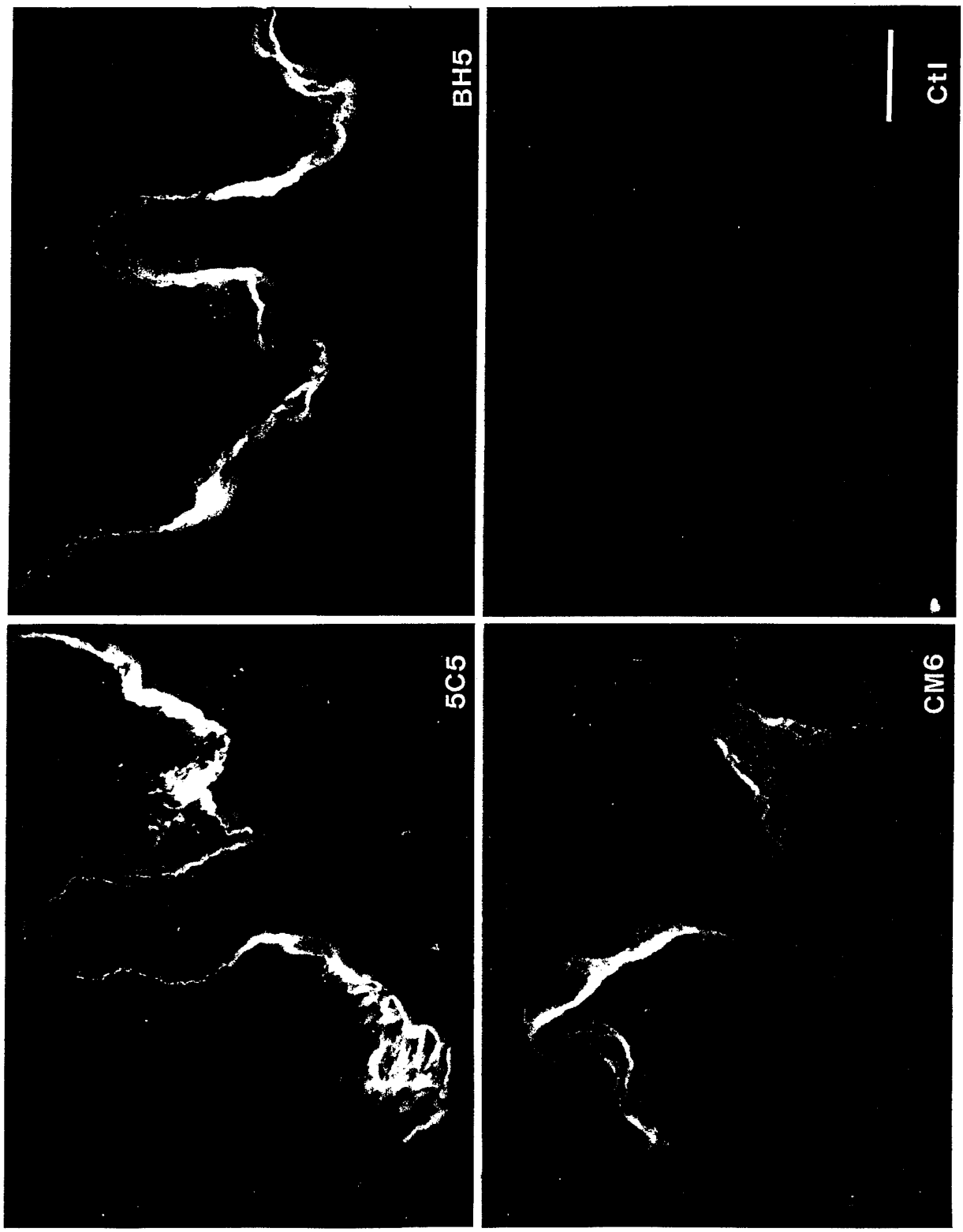
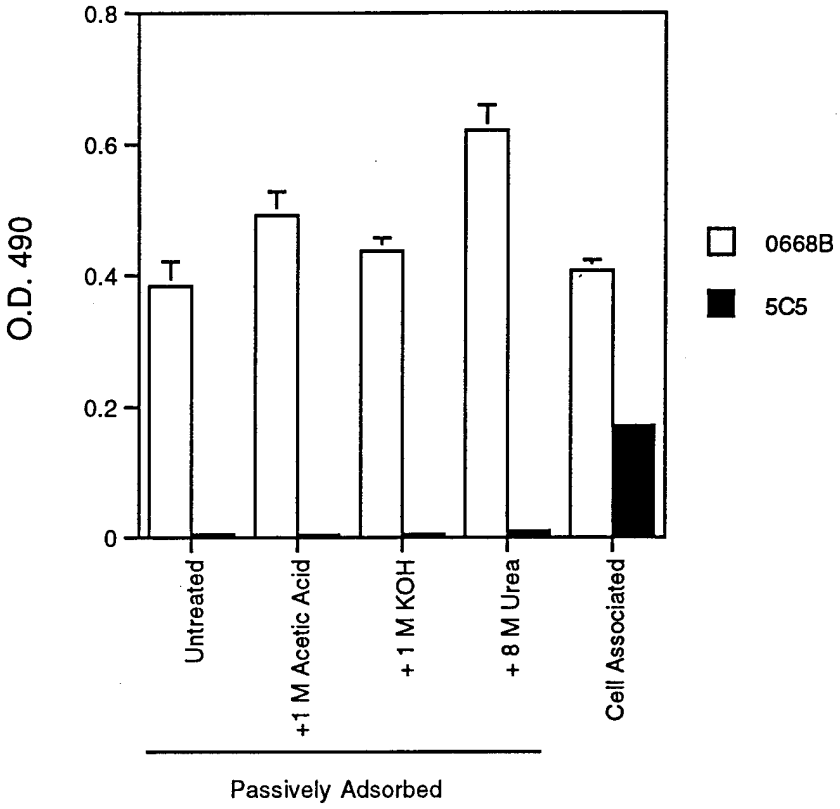


Figure 8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+1 M Acetic Acid
+ 1 M KOH
+ 8 M Urea
Cell Associated
Passively Adsorbed

Figure 9

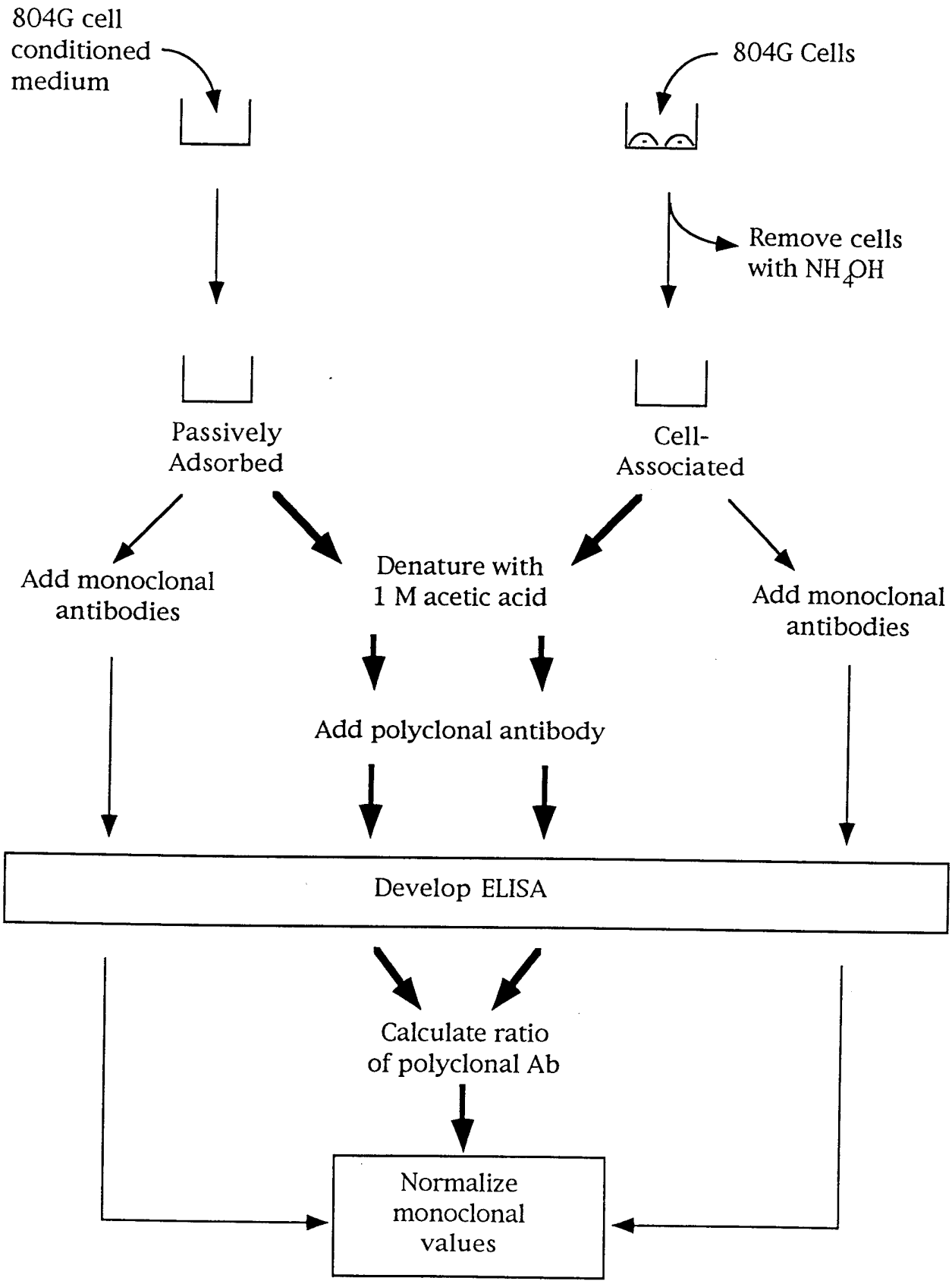
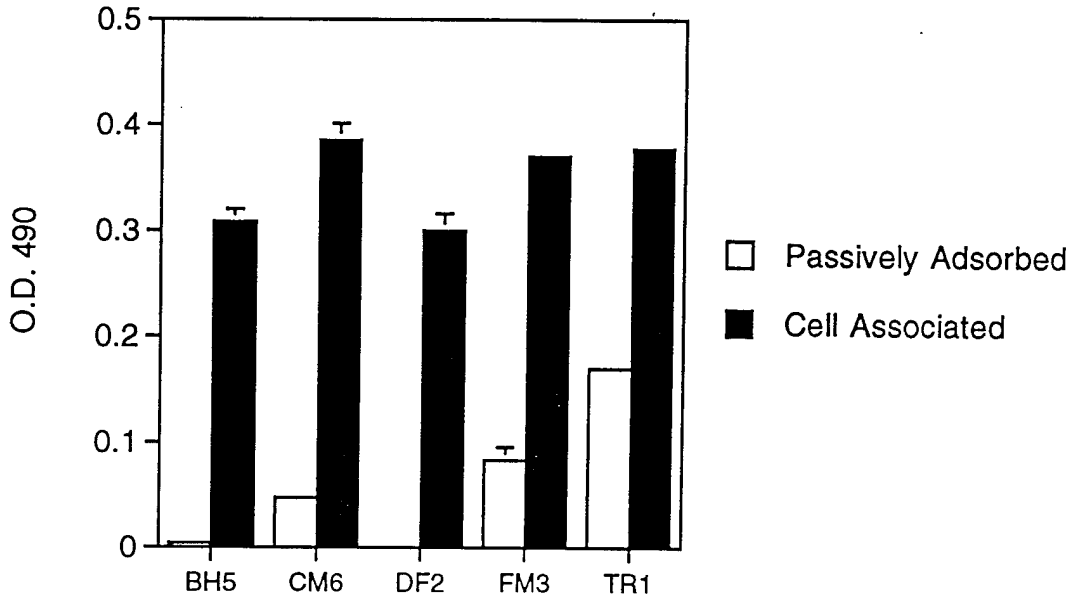

804G cell conditioned medium
804G Cells
Remove cells with NH_4OH
Passively Adsorbed
Cell-Associated
Add monoclonal antibodies
Denature with 1 M acetic acid
Add monoclonal antibodies
Add polyclonal antibody
Develop ELISA
Calculate ratio of polyclonal Ab
Normalize monoclonal values

Figure 10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BH5
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TR1
Passively Adsorbed
Cell Associated

Figure 11

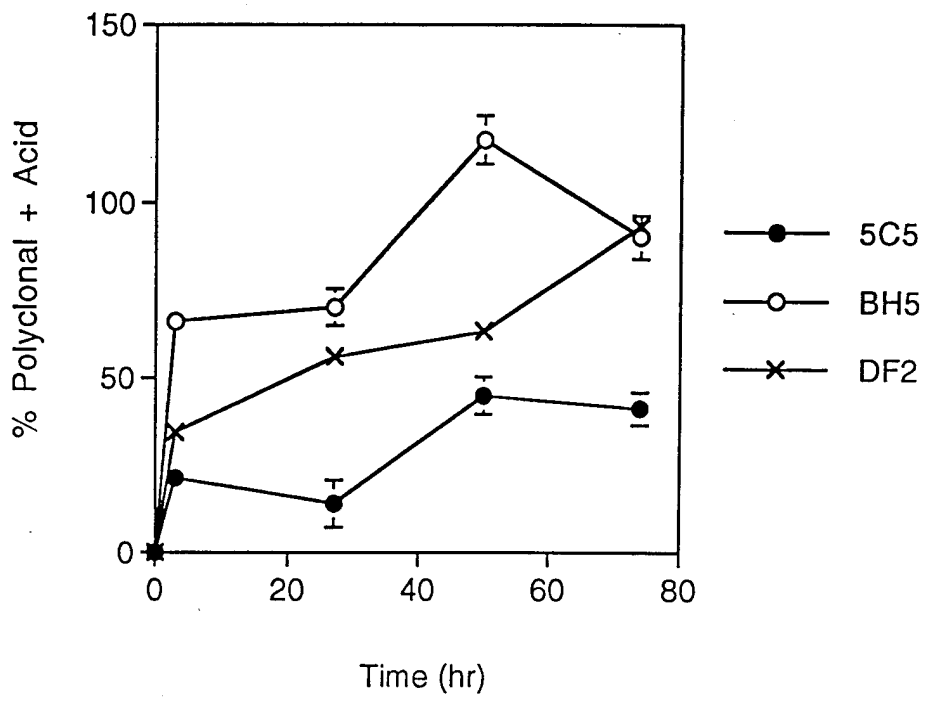
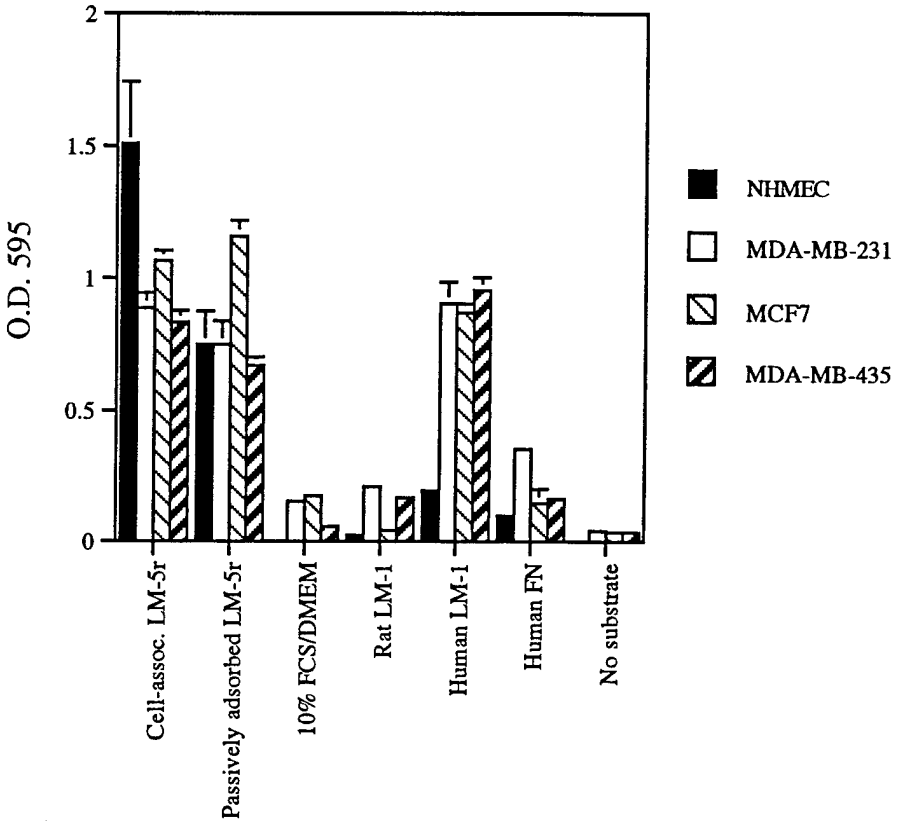


Figure 12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1.5
1
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0
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Passively adsorbed LM-5r
10% FCS/DMEM
Rat LM-1
Human LM-1
Human FN
No substrate
NHMEC
MDA-MB-231
MCF7
MDA-MB-435

Figure 13

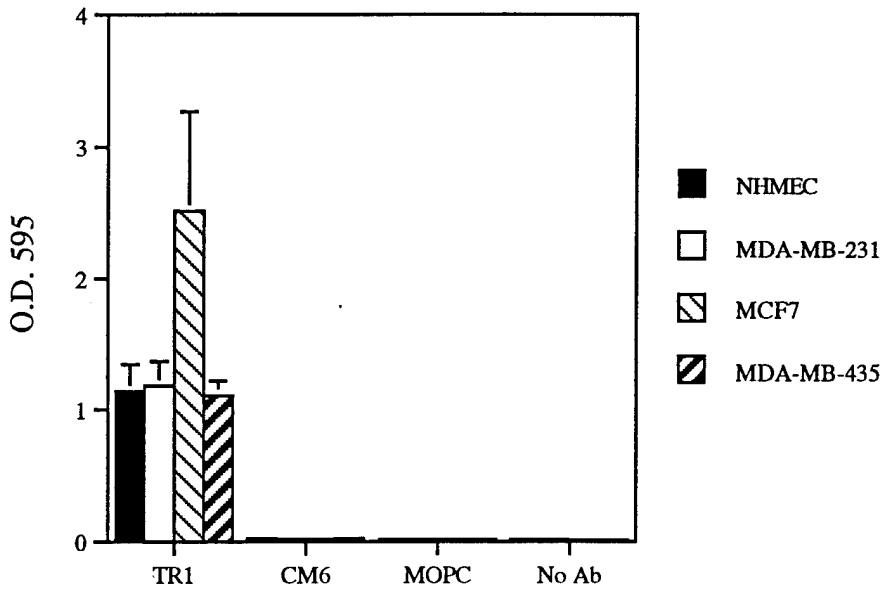
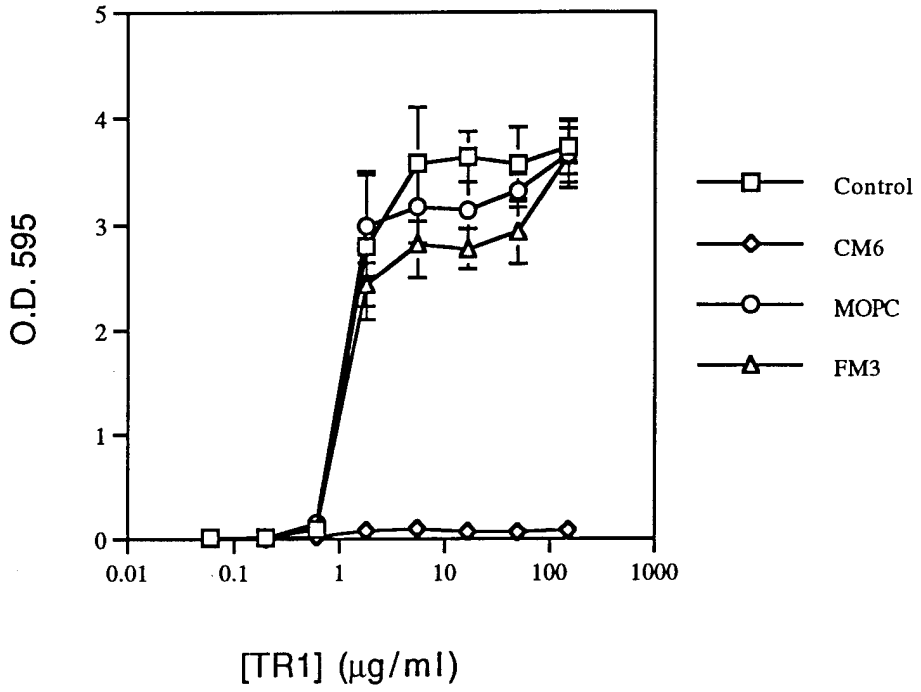


Figure 14


O.D. 595
5
4
3
2
1
0
0.01
0.1
1
10
100
1000
[TR1] (µg/ml)
Control
CM6
MOPC
FM3

Epitope	Soluble	Passively Adsorbed	Cell Associated
5C5	-	-	+
BH5	+	-	+
CM6	+	+	+
DF2	+	-	+
FM3	+	+	+
TR1	+	+	+

Table 1

Table 1. Surface epitope expression on three forms of laminin 5R. Epitopes on soluble laminin 5R were determined by immunoprecipitation. Epitopes on passively adsorbed and cell associated laminin 5R were determined by ELISA assay. Note that cell contact induces 5C5, BH5, and DF2 epitopes on passively adsorbed laminin 5R.